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TRANSMISSION OF MICROSPORIDIAN PARASITES OF MOSQUITOES
(U) FLORIDA UNIV GAINESVILLE DEPT OF ENTOMOLOGY AND
NEMATOLOGY D W HALL ET AL. JUN 83 N00014-80-C-0172

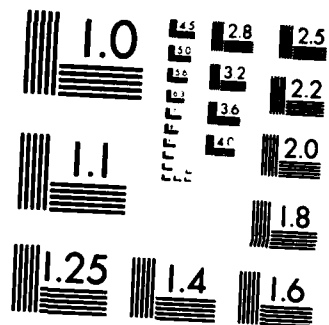
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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER Annual Report No. 3	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER 12
4. TITLE (and Subtitle) Transmission of Microsporidian Parasites of Mosquitoes		5. TYPE OF REPORT & PERIOD COVERED FINAL - Jan. 1, 1980 to June 31, 1983
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Donald W. Hall and Edwin I. Hazard		8. CONTRACT OR GRANT NUMBER(s) N00014-80-C-0172
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Entomology and Nematology University of Florida Gainesville, Florida 32611		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS NR 205-035
11. CONTROLLING OFFICE NAME AND ADDRESS		12. REPORT DATE June, 1983
		13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) Same		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Mosquito, <u>Culex</u> , Microsporidia, <u>Amblyospora</u> , horizontal transmission, intermediate host, ELISA, vertical transmission		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Life cycles of five species of microsporidian parasites of mosquitoes were studied. One of these has been described as a new species, <u>Microsporidium fimbriatum</u> . Extensive studies were conducted on the life cycle and host-parasite relationship of <u>Amblyospora</u> sp. in <u>Culex salinarius</u> . It was demonstrated that sporulation may be induced in the absence of a host blood meal by application of the hormone 20-hydroxyecdysone which is known to be secreted naturally by the ovaries after a blood meal. CONTINUED ON REVERSE SIDE...		

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20. ABSTRACT--continued.

The first enzyme-linked immunosorbent assay for a microsporidian was developed. The assay detects as little as 2 ng of spore homogenate protein and as few as 2000 intact spores. Several timesaving and reagent-conserving modifications of traditional ELISA protocols are employed.

Individuals of 103 taxa of aquatic animals were surveyed as possible intermediate hosts for Amblyospora. No successful transmissions were achieved with any of these animals.

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OFFICE OF NAVAL RESEARCH

Contract #N00014-80-C-0172

Task No. NR 205-035

FINAL TECHNICAL REPORT

Transmission of Microsporidian Parasites of Mosquitoes

by

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Microsporidia of the family Thelohaniidae are common parasites of mosquitoes and certain other invertebrates. They have complex life cycles and exhibit dimorphic development. In the adult female host the parasite forms small numbers of single binucleate spores which serve to infect the developing oocytes resulting in transovarial (vertical) transmission to the progeny of the infected female. Some species of microsporidia are transmitted in this manner for many generations while others are vertically transmitted for only 1 generation and all infected progeny die prior to reaching reproductive age. In both types of parasites a different type of spore is formed in the progeny than that formed in the infected female. These spores are uninucleate and packaged in groups of eight within a membrane. These uninucleate spores do not appear to be infectious when fed directly to mosquitoes.

We have shown that vertical transmission alone is not sufficient for maintenance of at least some of these parasites in nature. However, at the present time, none of these parasites have been successfully transmitted in the laboratory except by vertical transmission.

This contract is concerned with the microsporidia which have dimorphic life cycles and are transovarially transmitted in mosquitoes. The primary objectives of this research were to work out the life cycles of selected parasites and to determine the mechanism of horizontal transmission of the parasites from mosquito to mosquito.

This research was supported in part by the Office of Naval Research, Microbiology Program, Naval Biology Project, under Contract #N00014-80-C-0172, NR 205 - 035.

Life Cycle Studies.

Due to the extreme drought conditions in Florida during 1980 and 1981, we were unable to collect some of the desired mosquito species in sufficient numbers for life cycle studies of their microsporidian parasites. Consequently, work was concentrated on a new microsporidian species from the black salt-marsh mosquito *Aedes taeniorhynchus* which we have named *Microsporidium fimbriatum* and *Amblyospora* sp. from *Culex salinarius*. A short description of *M. fimbriatum* follows.

Microsporidium fimbriatum.

In the course of screening the progeny of female *Aedes taeniorhynchus* for transovarially transmitted pathogens egg batches infected with a microsporidium which resembles none previously described from mosquitoes were found.

Egg batches were obtained from individual mosquitoes collected in Everglades National Park by giving them a blood meal and placing them in vials containing gauze moistened with .15% NaCl. The resultant eggs were hatched and reared to screen for parasites. Screening involved examination of fourth instar larvae against a black background to detect discoloration and squashing emerged adults for examination with phase contrast microscopy. Some of the individuals from infected egg batches were smeared and stained with Giemsa, while others were prepared for electron microscopy.

Some eggs from 2 batches were infected with the microsporidium. In the first of these, 6 larvae developed patent infections in the terminal abdominal segments, most obviously in the fifth segment. One of these larvae was triturated and fed to 24 hr. old *A. taeniorhynchus* larvae which showed no patent infection when reared to the fourth instar, indicating a lack of *per os* transmission. The remainder of the larvae from the infected egg batch were reared

to the adult stage and examined for infection. Of the 16 adults that emerged, 12 females showed no sign of infection, but all 4 males contained numerous spores.

The second infected egg batch was hatched in two lots. The first lot was reared to the adult stage and found to contain one infected male. Smears were made with the female that laid the eggs and with the individuals from the second lot at intervals through their development and were stained with Giemsa. Three of these smeared individuals, a third instar larva, a fourth instar larva, and a pupa, were found to harbor the parasite. The smear of the parent female contained diplokaryotic stages of the parasite and empty cylindrical spore walls (Fig. 1) which appeared similar to the walls of the spores which function in transovarial transmission in members of the family Amblyosporidae (Hazard and Weiser, 1968; Andreadis and Hall, 1979a).

The third instar larva contained many diplokaryotic stages (Fig. 2,9) which are apparently the meronts (the primary multiplicative stages). They also contained a smaller number of binucleate and tetranucleate sporonts (Fig. 3), as well as a few scattered sporonts with six or eight nuclei (Fig. 4), sporoblasts (Fig. 5) and spores (Fig. 6,8). Sporogony continues through the fourth instar and pupal stage. Multinucleate sporonts appear to produce uninucleate sporoblasts by budding. There is apparently no pansporoblastic membrane, but this cannot be positively determined until it is possible to observe sporonts in electron micrographs.

Spores from larvae and adult males appear short pyriform with a sharp point at the anterior end when viewed in fresh smears. However, the point is difficult to demonstrate in Giemsa stained smears and electron micrographs (Fig. 5-8). The exospore is characterized by the presence of a fine, dense fringe.

Because insufficient information on its development and morphology make its taxonomic position unclear, we have assigned the species to the collective

genus *Microsporidium* suggested by Sprague (1977). Its development is most similar to dimorphic members of the family Amblyosporidae (Weiser, 1977) but the form of the spores found in males and the question of the pansporoblastic membrane preclude placement in this family at this time. We also do not know whether or not there is meiosis in the developmental sequence of the spores as has been reported for certain other microsporidia with dimorphic development (Hazard et al., 1979). The specific epithet, *fimbriatum*, means "fringed" in reference to the exospore of spores in male larvae.

Microsporidium fimbriatum sp.n.

Host. The black saltmarsh mosquito *Aedes taeniorhynchus* (Wiedemann).

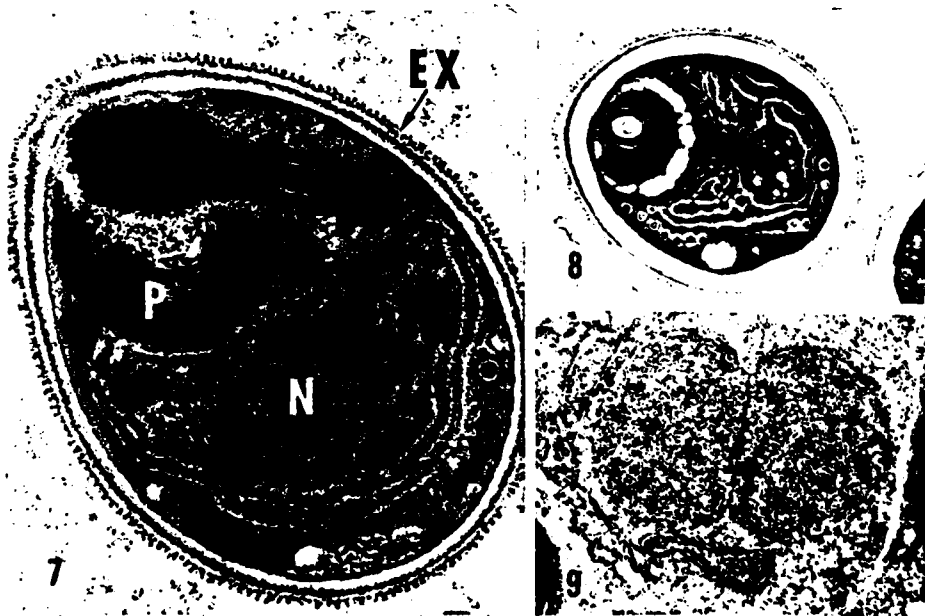
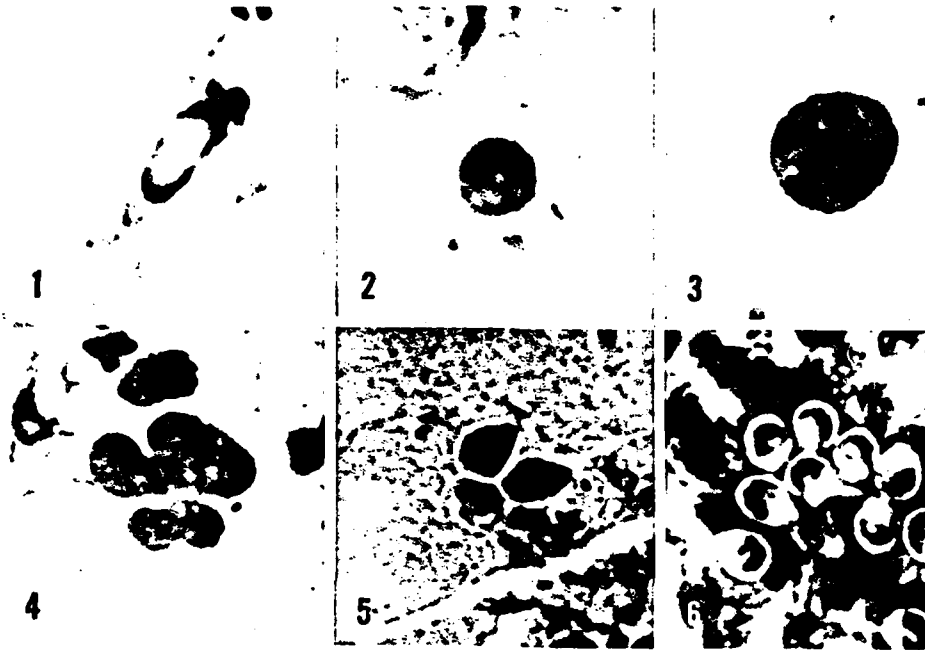
Type locality. Coastal Prairie Trail, Everglades National Park, Florida.

Vegetative stages. Meronts have one or two diplokarya.

Sporulation stages. Sporonts found in third and fourth instar larvae and pupae contain two, four, six, or eight nuclei. No pansporoblastic membrane is evident in the light microscope.

Spores. Two types of spores are produced, cylindrical spores in the adult female and uninucleate, short pyriform spores, having fringed exospores, in the last instar larvae, pupae, and adult males.

- Fig. 1. Empty spore case from adult female mosquito. Giemsa stain. X2400.
- Fig. 2. Binucleate meront from male larva. Giemsa stain. X2400.
- Fig. 3. Quadrinucleate sporont from male larva. Giemsa stain. X2400.
- Fig. 4. Octonucleate sporont from male larva. Giemsa stain. X2400.
- Fig. 5. Sporoblasts from male larva. Giemsa stain. X2800.
- Fig. 6. Mature spores from adult male. Giemsa stain. X2300.
- Fig. 7. Immature spore from adult male. EX, exospore; N, nucleus; P, polaroplast. X26,000.
- Fig. 8. Mature spore from adult male. X14,000.
- Fig. 9. Diplokaryotic stage in fourth instar larva. X15,000.



Amblyospora sp.

The general life cycle and quantitative aspects of vertical transmission of *Amblyospora* sp. in *Culex salinarius* has been worked out previously (Andreadis and Hall, 1979a, 1979b) (Fig. 10). However, there are still certain questions remaining regarding events in the adult female mosquito which lead to vertical transmission. Although there is a high efficiency of vertical transmission to progeny of infected females, over at least 5 gonotrophic cycles large numbers of infected oenocytes are never seen in Giemsa-stained smears of these mosquitoes. In an attempt to solve this puzzle, infected adult females of different ages were serially sectioned, and the total infected oenocytes were counted. These results are given in Table 1. Results for subsequent ovarian cycles are not yet tabulated. The average number of oenocytes per female for young females is 35.4. The number of parasites per oenocyte is variable but a realistic average might be 100. Based on an average of 327 eggs per female over 5 gonotrophic cycles, this provides for a potential multiplicity of infection of 11. An infected oenocyte is shown in Figure 10. It is evident that the oenocytes harbor a sufficient quantity of parasites to account for the observed rate of vertical transmission.

An interesting observation from the histological sections was that the infected oenocytes were somewhat randomly distributed throughout the bodies of the mosquitoes with a few even being found in the heads. Healthy larval oenocytes normally degenerate during the pupal stage and are restricted to the abdomen whereas infected ones persist in the adult stage and are freed to circulate in the hemocoel. Since the infected oenocytes are rarely adjacent to the ovaries, it is obvious that penetration of the ovaries by the polar filaments (the normal infection mechanism in *Micrasporidia*) is unlikely. It is probable that the sporoplasms are released through the polar filaments into the

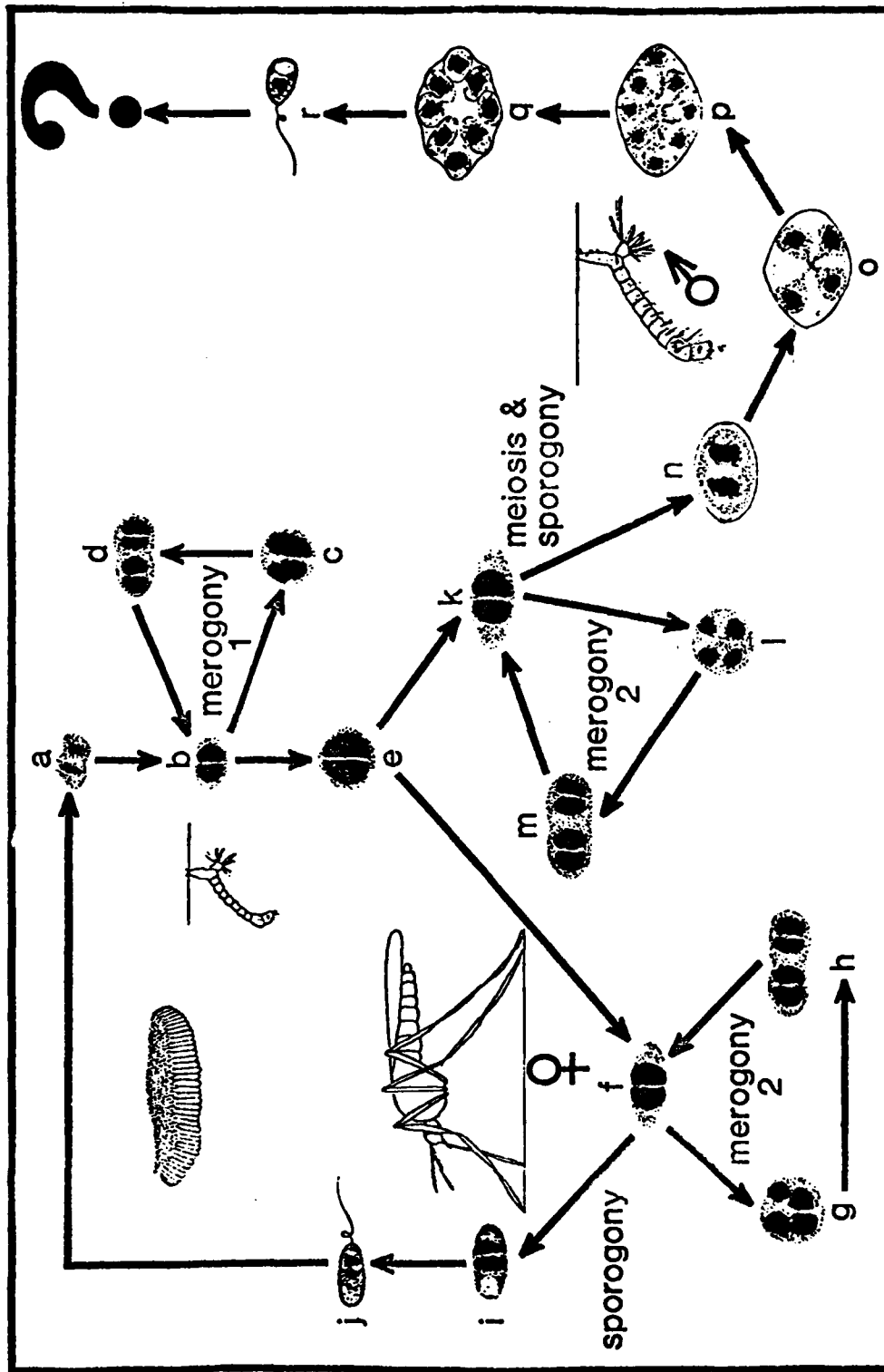


Fig. 10. Life cycle of *Amblyospora* sp. in *Cx. salinarius*. (a) Binucleate sporoplasm, (b-d) primary diplokaryon and stages of the 1st merogony in oenocytes of embryonated eggs and young larvae, (e) transitional diplokaryon, (f-h) secondary diplokaryon and stages of the 2nd merogony in oenocytes of newly emerged adult females, (i) sporoblast, (j) mature spore, (k-m) secondary diplokaryon and stages of the 2nd merogony in adipose tissue of young male larvae, (n) binucleate sporont, (o) quadrinucleate sporont, (p) otonucleate sporont, (q) pansporoblast containing eight sporoblasts, (r) mature haploid spore.

hemolymph and then carried to the ovaries where penetration occurs by an as yet unknown mechanism.

Another intriguing problem in the life cycle of *Amblyospora* sp. was the mechanism controlling sporulation in the adult female. The spores are formed synchronously with development of the ovarian follicles following a blood meal which is known to trigger the hormonal sequence leading to egg development. This suggests that the parasite may either respond to the nutrients released into the hemocoel from the blood meal or that there may be a more intimate host-parasite relationship in which the microsporidium responds to the physiological changes associated with the host's gonadotrophic cycle. In addition to blood meal nutrients the substances known to appear in the hemolymph during this cycle are vitellogenins and hormones, particularly egg development neurosecretory hormone (EDNH) (Lea, 1972) and ecdysteroids secreted by ovaries (Hagedorn et al., 1975). A study was conducted to determine which, if any, of these factors is used by *Amblyospora* sp. as a cue to initiate sporulation.

Treatments.

Healthy and *Amblyospora* infected adult female *C. salinarius* from laboratory colonies were used for experiments 5 and 6 days post-emergence. For all treatments mosquitoes were lightly anesthetized with nitrogen gas. Injections and topical applications were done with a finely drawn capillary tube calibrated to approximately 1 μ l. Cholesterol and 20-hydroxyecdysone were dissolved in insect saline with 10% ethanol. Methoprene and juvenile hormone (JH) I were dissolved in acetone. Egg macerate was prepared by removal of ovaries from gravid mosquitoes, maceration in a ground glass tissue grinder with 0.15 M NaCl buffered to pH 6.9, and centrifugation at ca. 800 g to remove particulate material.

Surgery.

To remove the medial neurosecretory cells and corpora cardiaca, the sources of EDNH, mosquitoes were decapitated, and the wound was sealed with paraffin. Decapitations of blood engorged individuals were done within 30 min. of feeding.

Ovariectomies were performed on mosquitoes starved for 24 hrs. prior to surgery. After placing them in a mold of modeling clay with thin strips of clay across the thoraxes as restrainers, the abdomens were immersed in saline. Sharpened jeweler's forceps were then used to remove the ovaries through single ventral incisions between the 6th and 7th abdominal sternites. Wounds were sealed with paraffin. Operated individuals were offered a guinea pig blood source after 6 hrs., then placed in humidified chambers.

Scoring.

Unless otherwise indicated all individuals were scored 48 hr. after treatment. Blood feeding of infected mosquitoes results in completion of sporulation after that length of time. Mature spores are then detectible for another 12-24 hr. (Andreadis and Hall, 1979a, 1979b).

Slides were prepared for screening for sporulation by smearing whole mosquitoes and staining with Giemsa stain after methanol fixation. Slides with a single spore were scored as positive. Those with at least 30 vegetative *Amblyospora* and no spores were scored as negative for sporulation. Spores and vegetative stages were easily distinguishable in Giemsa preparations (Fig. 11,12).

Results.

Decapitation of host mosquitoes immediately after blood feeding prevented the sporulation of *Amblyospora* that otherwise followed the blood meal (Table 2).

This indicates that factors other than nutrients from digested blood and gut stretch stimulus trigger the spore formation process. The presence of spores in 2 of the decapitated controls which were not given blood is probably due to autogeny which is present in a small percentage of the mosquitoes in our colony.

Since vitellogen appears in mosquito hemolymph shortly after a blood meal this protein must be considered as a candidate for the sporulation cue for *Amblyospora*. The vitellin of mature mosquito eggs is immunologically indistinguishable from its vitellogenin precursor (Hagedorn et al., 1978). With this in mind and with the consideration that other egg substances are presumably among those that appear in the hemolymph during gonadotrophic development it was decided to use the soluble material from a macerate of mature eggs for injection into infected *C. salinarius*. A 2-fold dilution of a concentration that proved lethal was used. In only 1 of 20 treated individuals were spores found (Table 3). Again the occurrence of autogeny may account for the presence of spores. Egg proteins appear not to induce sporulation of *Amblyospora*.

The approach taken to the question of hormonal induction of sporulation was to treat infected mosquitoes with the hormones and their analogs. Since EDNH is not commercially available, indeed its exact nature is unknown, this hormone could not be used.

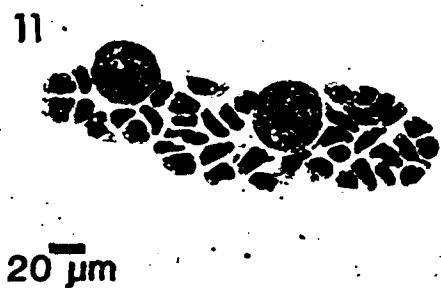
JH is required for the development of mosquito ovaries to the previtellogenic stage (Lea, 1963, 1969). Borovsky (1981) has speculated on a vitellogenic role for this hormone. To determine if it has an effect on *Amblyospora*, JH I and its more stable analog methoprene were applied topically to infected *C. salinarius*. JH I was ineffective at 1 μ g, and methoprene was ineffective up to a dose of 6 μ g which was lethal to most mosquitoes (Table 4).

Shortly after a mosquito takes a blood meal its ovaries secrete ecdysone, which is then hydroxylated by other tissues to form the more active 20-hydroxyecdysone. This hormone, when injected into infected mosquitoes at low doses, did not induce sporulation. At a dose of 2.5 μ g all 32 of the individuals injected contained spores (Table 5). This is a pharmacological rather than a physiological dose. The fact that such a large dose is required may be explained, at least in part, by the rapid degradation of ecdysteroid in vivo (Ohtaki and Williams, 1970). It seems likely from these results that 20-hydroxyecdysone, or some substance whose presence is induced by it, is the cue to which *Amblyospora* responds by sporulating.

To see if this was a general response to steroids cholesterol was injected into infected *C. salinarius*. This was without effect (Table 6).

Since the ovaries are the source of ecdysteroids in blood-fed mosquitoes we decided to determine whether *Amblyospora* would sporulate in mosquitoes which were given blood after the removal of their ovaries. In none of those so treated did sporulation occur (Table 7). 20-hydroxyecdysone induces synthesis of dopa decarboxylase in insects. This enzyme catalyzes production of catecholamines involved in sclerotization of the insect cuticle and presumably the egg chorion. Furthermore the microsporidian spore wall resembles the cuticle in being of protein and chitin (Vavra, 1976). To see if catecholamines appearing in the hemolymph following 20-hydroxyecdysone are a sporulation trigger, dopamine and N-acetyldopamine were injected into the host. Neither was effective (Table 8).

Finally, to determine if the spores formed in response to 20-hydroxyecdysone are viable, electron micrographs were prepared from hormone injected and blood-fed mosquitoes. There were no discernible differences in the ultrastructure of the resultant sporoblasts.



Photomicrographs of *Amblyospora* sp. in syncytial oenocytes of adult female *C. salinarius* (Giemsa stain).

- 11. Diplokaryotic vegetative stages
- 12. Spores

TABLE 1. TOTAL OENOCYTE COUNTS FROM *Amblyospora*-INFECTED NULLIPAROUS ADULT FEMALE *Culex salinarius**

4-7 days old (non-blood-fed)	>10 days old (non-blood-fed)	>21 days old (77 hr. post-blood-feeding)
50	39	29
33	23	30
27	24	21
22	32	16
12	11	37
42	13	18
56	31	10
41	44	30
	28	28
	50	

*Age refers to time since adult eclosion.

TABLE 2. EFFECT OF DECAPITATION OF NEWLY BLOOD-FED *C. salinarius* ON SPORULATION OF *Amblyospora* sp.

	<u>Vegetative stages only</u>	<u>Spores</u>
Blood-fed, decapitated	24	0
Blood-fed, not decapitated	0	20
No blood, decapitated	14	2

TABLE 3. EFFECT ON SPORULATION OF *Amblyospora* sp. OF INJECTION OF HOST EGG MACERATE INTO *C. salinarius*

	<u>Vegetative stages only</u>	<u>Spores</u>
Egg macerate	19	1
Saline	16	0

TABLE 4. EFFECT ON SPORULATION OF *Amblyospora* sp. OF TOPICAL APPLICATION OF JH I AND METHOPRENE TO *C. salinarius*

	<u>Vegetative stages only</u>	<u>Spores</u>
10 ng methoprene	20	0
60 ng methoprene	30 (5 at 72 hr)	0
1 µg methoprene	18	0
6 µg methoprene	12 (5 at 72 hr)	0
1 µg JH I	32	0
Acetone	14	0

TABLE 5. EFFECT ON SPORULATION OF *Amblyospora* sp. OF INJECTION OF 20-HYDROXYECDYSONE INTO *C. salinarius*

	<u>Vegetative stages only</u>	<u>Spores</u>
100 pg + 250 pg at 15 hr	8	0
10 ng	14	0
2.5 µg	0	32
Saline	28	0

TABLE 6. EFFECT ON SPORULATION OF *Amblyospora* sp. OF INJECTION OF CHOLESTEROL INTO *C. salinarius*

	<u>Vegetative stages only</u>	<u>Spores</u>
2.5 µg Cholesterol	16	0
Saline	12	0

TABLE 7. EFFECT ON SPORULATION OF *Amblyospora* sp. OF OVARIECTOMY OF *C. salinarius*

	<u>Vegetative stages only</u>	<u>Spores</u>
Ovariectomized and blood-fed	26	0
Sham-operated and blood-fed	1	16

TABLE 8. EFFECT ON SPORULATION OF *Amblyospora* sp. OF INJECTION OF CATECHOLAMINES INTO *C. salinarius*

	<u>Vegetative stages only</u>	<u>Spores</u>
10 µg Dopamine	16	0
1.5 µg N-acetyldopamine	23	2
Saline	20	0

In summary it is concluded that 20-hydroxyecdysone induces sporulation of *Amblyospora* in *C. salinarius*. Whether the parasite responds directly to the hormone or to some substance that appears in the hemolymph as a result of the hormone's presence has yet to be determined.

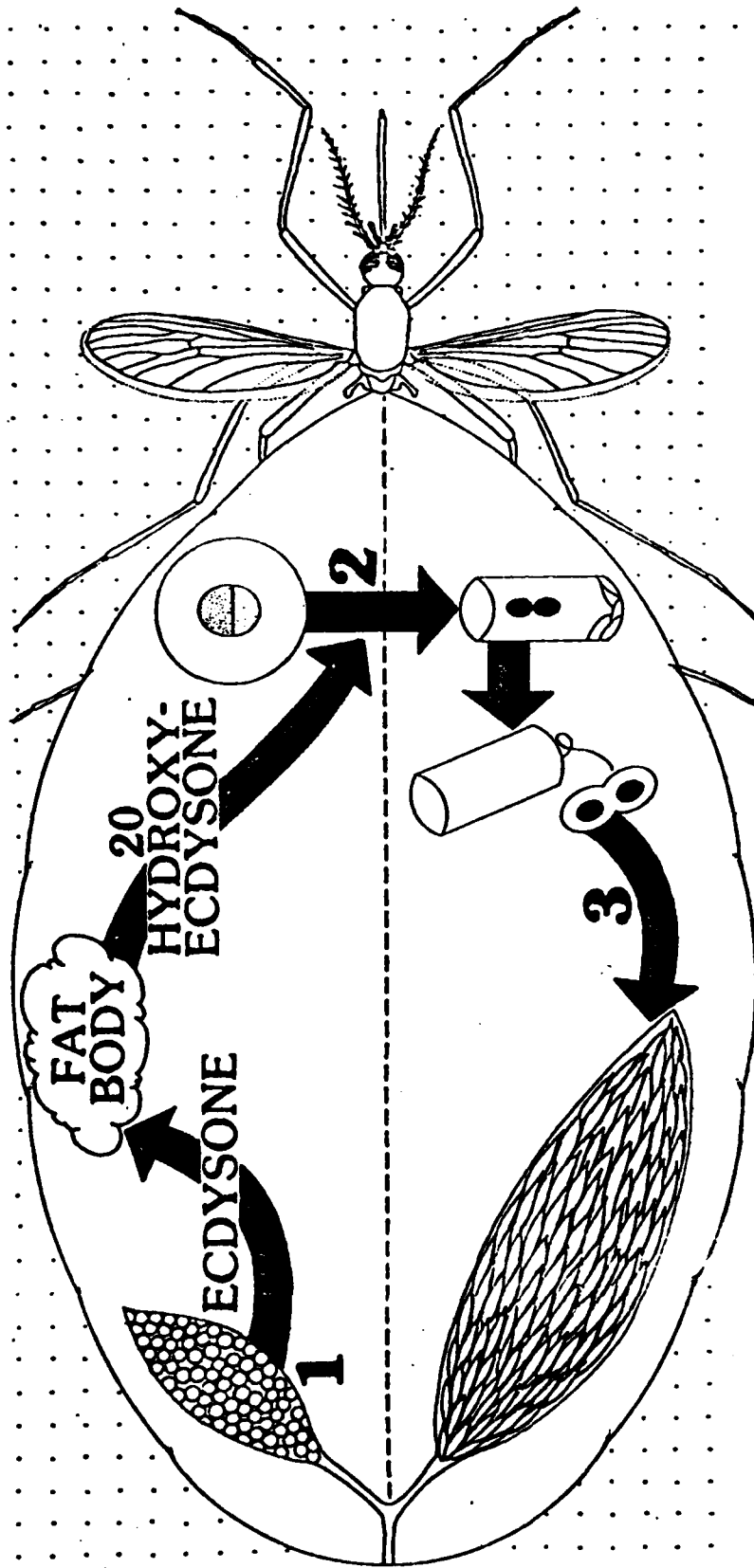
A proposed scheme for sporulation and subsequent infection of the developing eggs is presented in Figure 13.

Horizontal Transmission Studies.

Since the haploid spores formed in large numbers in male *C. salinarius* larvae do not appear to be infective when fed back to healthy larvae, we have hypothesized the existence of an intermediate host. Several approaches are possible to test this hypothesis. The first is to feed the spores to candidate intermediate hosts and look for signs of development of the parasite. It is also possible that the spores may be conditioned by passing through the digestive tracts of other organisms. Studies to investigate these possibilities have been done.

Spore Feeding Experiments.

To survey possible intermediate hosts of *Amblyospora* sp., pairs of jars were used, an experimental jar and an assay jar, each containing about 500 ml of aged tap water. The experimental jar contained one to many individuals of a candidate intermediate host species, with a piece of aluminum screening or a thin layer of sand for those species that required a solid substrate. Predators were fed live infected *C. salinarius* (containing haploid spores) larvae, scavengers were fed mashed infected *C. salinarius* larvae and Koi Goldfish Food. The assay jar contained healthy *C. salinarius* larvae. Five healthy first instar larvae were added each day to each assay jar, since young



- 1** BLOOD MEAL TRIGGERS OVARIAN SECRETION OF ECDYSONE
- 2** SPORULATION IS COMPLETED BY 48 HR. POST BLOOD MEAL
IN RESPONSE TO 20 HYDROXYECDYSONE
- 3** SPOROPLASM INFECTS DEVELOPING EGGS

Figure 13.

larvae are probably more easily infected *per os* than older larvae. About 1.5 ml of water from the bottom of each experimental jar, hopefully containing some infective *Amblyospora* stage, were transferred daily to the appropriate paired assay jar. Both jars were gently stirred daily to suspend at least briefly any infective stages in the water column. Fourth instar *C. salinarius* in the assay jar were examined for the white coloration due to an overt infection of *Amblyospora* sp. To check for transovarial infections, adult *C. salinarius* were reared from the assay jars, the females fed blood and allowed to oviposit, the larvae reared until pupation began, and the second generation fourth instar larvae examined for overt infections.

A total of 142 replicate jar pairs, each containing one to many individuals of more than 103 taxa of aquatic animals, were surveyed (Table 9). These totals exclude Protozoa, Rotatoria, and Nematoda, which were present in most or all of the jars. From the assay jars, more than 1341 female and 1435 male fourth instar *C. salinarius* larvae were reared and examined for overt infections (sex determined at adult emergence). None of the larvae showed *Amblyospora* infections. From this generation of adult female *C. salinarius*, more than 1291 egg rafts were produced, and a second generation larvae also showed no infections.

The following should also be noted: 1) *Amblyospora* spores had a chance to infect the healthy *C. salinarius per os* in all assay jars, but did not cause infections, 2) Aging of the spores for up to 2-4 months did not produce infectivity, 3) Passage of the spores through the guts of the experimental animals, probably many times, did not produce infectivity, and 4) No infections from microsporidians present in the wild-caught hosts were detected in the assay *C. salinarius* larvae.

Miscellaneous experiments-- Various attempts were made to cause *Amblyospora* spores to germinate, including: 1) Crushing tissues of aquatic animals in a

spore suspension, including ostracods, amphipods, spiders, beetle larvae, and phantom midges, 2) Feeding spores to crayfish, dragonfly larvae, damselfly larvae, water scorpions, beetles, *Anopheles* larvae, snails, and mosquitofish. In experiment 1, no germination was seen. In experiment 2, the spores usually did not germinate, but 7 germinated spores were found in the gut of a *Pantala flavescens* dragonfly larvae 2 hrs after feeding. Cuts of some of the other animals had empty spore walls or dissociated polar filaments. However, no infections from any of these hosts were produced in the assay experiment, and the germination experiments were abandoned.

Two other experiments were attempted: 3) Rearing healthy *C. salinarius* larvae from egg to fourth instar larvae in water from 3 different ponds, and 4) Rearing healthy *C. salinarius* in water containing *Amblyospora* spores at 38 deg. C. No overt infections were found in full grown *C. salinarius* larvae in either of these experiments.

TABLE 9. ANIMALS TESTED AS INTERMEDIATE HOSTS OF *Amblyospora* sp. in *Culex salinarius*. AN ASTERISK (*) INDICATES A PROBABLY INADEQUATE TEST. A (+) INDICATES MANY REPLICATES OR INDIVIDUALS. FOR LIFE STAGES, A = ADULT; I = IMMATURE, i.e., JUVENILE, LARVA, NYMPH.

Taxon	Number of Replicates	Number of Individuals	Life-Stage
PROTOZOA			
Mastigophora	115	+	A
Sarcodina	1	+	A
Ciliata	115	+	
PORIFERA	1	3	A
PLATYHELMINTHES			
Turbellaria	3	+	AI
ROTATORIA	+	+	AI
NEMATODA	+	+	AI
NEMATOMORPHA	1	2	A
ANNELIDA			
Oligochaeta	2	9	A
ARTHROPODA			
Crustacea			
Cladocera	2	+	A
Copepoda	3	+	AI
Ostracoda	7	+	AI
Amphipoda			
<i>Hyalella azteca</i>	1	+	AI
Decapoda			
<i>Palaemonetes</i> sp.	1	3	AI
<i>Procambarus fallax</i>	3	12	I
Arachnida			
Hydracarina	1	+	AI
Araneida			
<i>Dolomedes</i> sp.	2	2	I
<i>Pisaurina mira</i> *	1	1	I
Insecta			
Ephemeroptera			
<i>Caenis</i> sp.	1	2	I
<i>Callibaetis</i> sp.	1	3	I
<i>Centroptilum hobbisi</i> *	1	2	I
<i>Hexagenia munda</i>	1	2	I
<i>Stenacron interpunctatum</i>	2	13	I
Odonata			
<i>Calopteryx maculata</i>	1	+	I
<i>Anomalagrion hastatum</i>	1	+	I
<i>Argia sedula</i>	2	9	I
<i>Enallagma cardenium</i>	1	2	I

TABLE 9.--continued.

Taxon	Number of Replicates	Number of Individuals	Life-Stage
Odonata--continued.			
<i>E. signatum</i>	2	6	I
<i>Ischnura posita</i>	1	4	I
<i>I. ramburii</i>	3	+	I
<i>Anax longipes</i>	2	3	I
<i>Epiaeschna heros</i>	2	2	I
<i>Nasiaeschna pentacantha</i>	1	1	I
<i>Gomphus minutus</i>	1	5	I
<i>Progomphus obscurus</i>	1	2	I
<i>Macromia georgina</i>	1	1	I
<i>Tetragoneuria</i> sp.	1	1	I
<i>Erythemis simplicicollis</i>	1	11	I
<i>Libellula</i> sp.	1	3	I
<i>Pachydiplax longipennis</i>	1	7	I
<i>Pantala flavescens</i>	2	16	I
<i>Perithemis tenera</i>	1	1	I
<i>Tramea</i> sp.	1	4	I
Hemiptera			
<i>Hydrometra martini</i> *	1	6	AI
<i>Mesovelis mulsanti</i>	1	9	AI
<i>Gerris</i> sp.*	1	3	AI
<i>Microvelis</i> sp.	1	10	AI
<i>Buenoa margaritacea</i> *	1	1	A
<i>Notonecta indica</i>	2	7	AI
<i>N. irrorata</i>	1	1	A
<i>Plea striola</i>	1	4	AI
<i>Ranatra nigra</i>	1	7	AI
<i>Belostoma lutarium</i>	1	3	A
<i>B. testaceum</i>	3	8	AI
<i>Lethocerus griseus</i> *	1	1	A
<i>Corixidae</i> sp.*	1	4	A
Megaloptera			
<i>Corydalus cornutus</i>	1	2	I
<i>Sialis americana</i>	1	1	I
Trichoptera			
<i>Chumatopsyche</i> sp.	1	8	I
<i>Hydropsyche</i> sp.	1	7	I
Coleoptera			
<i>Peltodytes oppositus</i>	1	12	A
<i>Acilius semisulcatum</i>	1	4	A
<i>Anodochilus exiguus</i>	1	6	A
<i>Cryptotomus interrogatus</i>	1	8	A
<i>Desmopachria mutchleri</i>	1	1	A
<i>Hydroporus falli</i>	1	3	A
<i>Laccophilus fasciatus</i>	1	6	A
<i>L.</i> sp.	1	4	A
<i>Dytiscidae</i> sp.	3	5	AI
<i>Dineutus carolinus</i>	1	5	A
<i>Berosus exiguus</i>	1	10	A

TABLE 9.--continued.

Taxon	Number of Replicates	Number of Individuals	Life-Stage
Coleoptera--continued.			
<i>B. infuscatus</i>	1	5	A
<i>Enochrus sublongus</i>	2	4	A
<i>Hydrochara</i> sp.	1	2	A
<i>Tropisternus lateralis</i>	1	5	A
Hydrophilidae sp.	1	7	I
<i>Helichus striatus</i>	1	1	A
<i>Dubiraphia quadrinotatus</i>	1	3	AI
<i>Elodes</i> sp.	1	+	I
Diptera			
<i>Telmatoscopus superbus</i>	1	22	I
Psychodidae sp.	7	+	I
<i>Aedes aegypti</i>	1	+	I
<i>A. triseriatus</i>	1	+	I
<i>Anopheles quadrimaculatus</i>	1	+	I
<i>Culex pilosus</i>	1	4	I
<i>C. territans</i>	1	4	I
<i>Chaoborus</i> sp.	1	22	I
Simuliidae sp.	1	28	I
Chironomidae sp.	1	+	I
<i>Culicoides mississippiensis</i> *	2	+	I
<i>Hedriodiscus trivittatus</i>	1	13	I
Muscidae sp.	1	9	I
MOLLUSCA			
Gastropoda			
<i>Goniobasis</i> sp.	1	9	A
<i>Gyraulus</i> sp.	2	+	AI
<i>Physa</i> sp.	2	+	AI
<i>Planorbella scalare</i>	1	+	AI
<i>Pomacea</i> sp.	3	+	I
Pelecypoda			
<i>Corbicula manilensis</i>	2	5	AI
<i>Pleurobema pyriforme</i>	2	5	AI
PISCES			
<i>Gambusia affinis</i>	3	9	AI
<i>Chaenobryttus coronarius</i>	1	1	I
<i>Lepomis marginatus</i>	1	1	I
AMPHIBIA			
	<u>1</u>	<u>2</u>	I
	142+	438+	

Serological Studies.

Another approach to searching for candidate intermediate hosts is the use of specific antibodies to *Amblyospora* in ultrasensitive serological assays of wild collected aquatic invertebrates. Final work on an enzyme-linked immunosorbent assay (ELISA) was completed. A description of this technique follows. Infected fourth instar larvae were harvested, rinsed in tap water, macerated in a tissue grinder, and stored at -80°C in phosphate-buffered saline (PBS), pH 7.4, until sufficient number had been accumulated for efficient purification. Thawed macerates were centrifuged for 2 min at 600g to remove large mosquito fragments. Spores were then concentrated by centrifugation for 5 min at 700g and layered onto a 0-20% Ludox HS-40 (E.I. du Pont de Nemours, Wilmington, Del.) continuous density gradient (Undeen and Alger, 1971) and centrifuged for 4 hr at 1500g at 4°C . After four washes in large volumes of PBS, pH 7.4, the spores were counted with a hemacytometer and stored at -80°C until needed.

Spore homogenates were prepared by combining thawed spore suspensions at 10^8 /ml with an equal volume of 0.5-mm glass beads and homogenizing in a Braun MSK tissue homogenizer (Knell, 1975). Homogenates were checked with the hemacytometer to ensure at least 95% spore disruption. Homogenate protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.), using bovine serum albumin (BSA) (Fraction V powder, Sigma, St. Louis, Mo.) as control.

Two 25-g BALB/c mice received intraperitoneal injections of 100 μg homogenate protein each in PBS, pH 7.4, emulsified with an equal volume (0.35 ml) of Freund's complete adjuvant. At intervals of 2 to 3 weeks they received two additional injections of 100 μg homogenate protein without adjuvant. One month following the final injection they were bled from the ventral tail vein and

their blood was pooled. Blood was allowed to clot for 1 hr and the serum was removed and heat inactivated (Kabat and Mayer, 1961). Immunoglobulin was precipitated with 38% ammonium sulfate, dissolved in and dialyzed three times against large volumes of PBS, pH 7.4, and stored at a 1:5 dilution at 4°C prior to use.

Indirect ELISA's (Voller et al., 1976) were performed, using sheep anti-mouse immunoglobulin, kindly furnished by G.A. Gutman, as second antibody. The sheep antibody was conjugated to alkaline phosphatase (Sigma) according to the protocol of Clark and Adams (1977).

In order to determine optimum reagent concentrations, checkboard titrations were set up using all combinations of antigen at 10, 3.3, 1.1, 0.37, and 0.12 µg/ml, mouse anti-*Amblyospora* immunoglobulin at 10, 3.3, 1.1, 0.37, 0.12, 0.04, 0.014, and 0.005 µg/ml, and sheep anti-mouse immunoglobulin conjugate at dilutions of 1:100, 1:500, and 1:1,000.

All assays were performed in 96-well flat-bottomed Linbro EIA plates (Flow Laboratories, McLean, Va.), using 0.1-ml quantities of reagents. Plates were coated with antigen in PBS, pH 7.4, or carbonate buffer, pH 9.6, (Clark and Adams, 1977), incubated 1 hr at 37°C, and washed four times for 3 min each with PBS-Tween (PBST) (Clark and Adams, 1977). Following this mouse anti-*Amblyospora* immunoglobulin was introduced in PBST with 0.05% BSA (PBST-BSA), incubated 1 hr at 37°C and unbound immunoglobulin removed by washing. Next, sheep anti-mouse immunoglobulin conjugate in PBST-BSA was introduced and the plate incubated and washed as before. Finally, substrate (p-nitrophenyl phosphate, Sigma, 1 mg/ml) in diethanolamine buffer, pH 9.8 (Clark and Adams, 1977), was added, incubated for 1 hr at room temperature, and the reaction stopped by the addition of 0.05 ml of 3 M NaOH. Six- or ten-replicate buffer and normal mouse serum (NMS) controls were run with each assay. The absorbance at 405 nm

of each well was determined with a Titertek Multi-Skan 8-Channel photometer (Flow Lab.).

Ten-replicate titrations of spore homogenate at 50, 30, 15, 5, and 2 ng protein per well, and of intact spores at 40,000, 20,000, 10,000, and 5,000 per well, were performed. Homogenate titrations were assayed as described above; spore titration plates were coated and incubated overnight at 4°C rather than 1 hr at 37°C.

The relationships between homogenate protein mass, *Amblyospora* spore number, and absorbance at 405 nm were determined by weighted regression analysis (Draper and Smith, 1981, p. 108). The sensitivities of the assays were determined by inverse regression (Draper and Smith, 1981, pp. 47-50), and expressed as the largest value of homogenate mass or spore number for which the estimated absorbance differs significantly from that of the mean of the control absorbences. This method is preferable to the usual approach of comparing just the means of the control and the lowest concentration of material tested by the t test, because it uses information from the entire titration (50 or 40 observations) rather than just those from the single lowest concentration (10 observations). Sensitivity to homogenate protein is expressed in mass rather than concentration because the photometer absorbences are directly proportional to mass of material (Titertek manual, Flow Lab.).

The assay was most sensitive using mouse anti-*Amblyospora* immunoglobulin at 3.3 µg/ml and sheep anti-mouse immunoglobulin conjugate at 1:100 dilution. Assays using PBS and carbonate-coating buffers did not differ in sensitivity so the former was adopted for all subsequent work. *Amblyospora* homogenate masses greater than 50 ng/well and intact spore numbers greater than 50,000/well gave off-scale readings on the photometer.

Data from the *Amblyospora* homogenate and spore titrations are shown in Figures 14 and 15, respectively. The equation

$$y = 0.017x + 0.156$$

explains 97% of the total weighted variation in Figure 14, and

$$y = 27.3x + 0.062$$

explains 99% of that in Figure 15. The estimated sensitivities of these two assays are 1.57 ng homogenate protein and 1710 spores, respectively ($P < 0.05$, one-sided test).

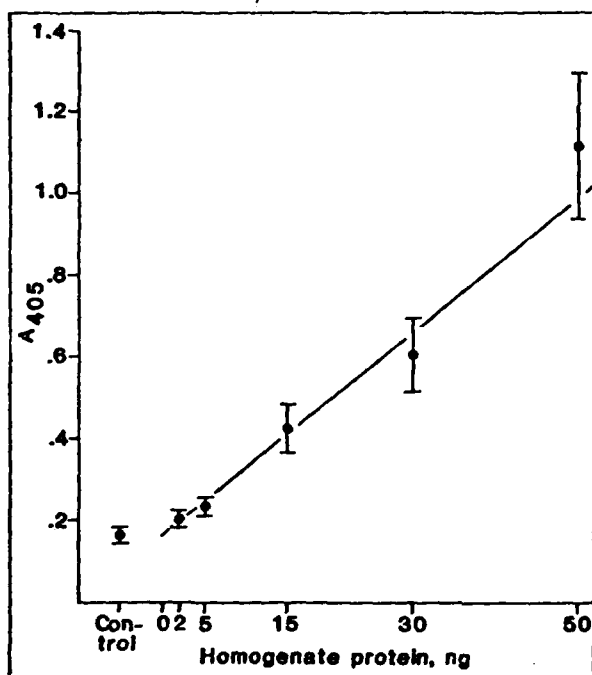


Figure 14. The relationship between *Amblyospora* homogenate protein mass and absorbance at 405 nm in the indirect ELISA. Data shown are means and 95% confidence intervals for ten observations at each mass; line was fitted by weighted linear regression (Draper and Smith, 1981).

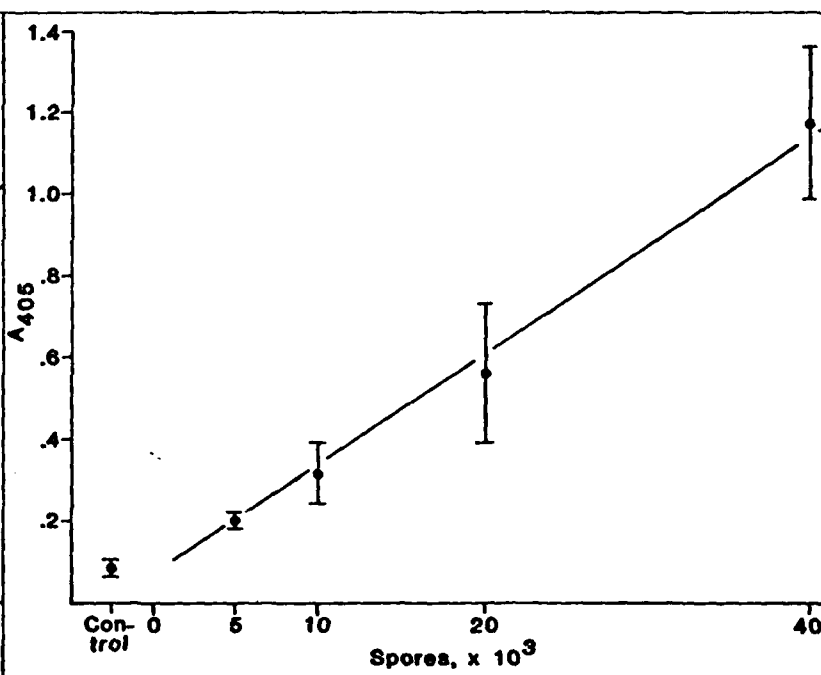


Figure 15. The relationship between *Amblyospora* spore number and absorbance at 405 nm in the indirect ELISA.

The indirect rather than the more popular double antibody sandwich (Voller et al., 1976; Clark and Adams, 1977) ELISA configuration was initially chosen because the assay will eventually be performed using monoclonal antibodies (Kohler and Milstein, 1975) and the indirect configuration lends itself better to a screening assay for hybridoma antibody production. (It also explains the use of mice rather than rabbits). However in practice the indirect ELISA assay has usually proven to be about ten times more sensitive in assays for antigen (Kaupp, 1980; Crook and Payne, 1980). It has the additional advantage that whereas specific antibody may be in short supply, particularly in the case of antibodies to microbial pathogens which are themselves precious, second antibodies are generally abundant, and may even be purchased commercially as enzyme conjugates. This essentially doubles the usable supply of specific antibody and also makes it possible to assay for several different antigens using specific antibodies produced in the same vertebrate species without having to make a different conjugate for each.

The procedure described here differs from traditional protocols in three other respects. First, the use of the photometer, which reads directly through the wells of the EIA plate, obviates the need to dilute and read each sample separately, speeding up the process and making it possible to reduce the reagent volume to 0.1 ml, the minimum required to coat the plate [plates with half-volume (0.05 ml) wells are also becoming available]. Second, reaction times have been drastically shortened (cf. Korpraditskul et al., 1979) so that a complete assay, including washes and reading, can be run in less than 6 hr. Since each plate has 60 usable wells (the outer wells of EIA plates tend to give discordant readings), hundreds of assays can be run in a day. Finally, PBS, pH 7.4, was substituted for carbonate, pH 9.6, as the coating buffer. This eliminates the need to make up the unstable carbonate buffer fresh each week and reduces the

chances of adding a reagent in the incorrect buffer. (Crook and Payne, 1980, found that their anti-baculovirus ELISA was actually more sensitive with the PBS-coating buffer.)

This is the first ELISA reported for a microsporidian. Its sensitivity for detecting antigen protein is comparable to that of ELISA assays insect viruses (Kelly et al., 1978a, 1978b; Kaupp, 1980; Crook and Payne, 1980; Longworth and Carey, 1980). Aside from the immediate goal of identifying intermediate hosts of *Amblyospora* sp., assays like this one are essential for epidemiological studies of microsporidia occurring as natural epizootics or in biological control programs. For example the ability to detect fewer than 2000 spores will make it possible to identify prepatent infections in young *C. salinarius* larvae.

Life Cycle Studies.

The life cycles of 3 additional microsporidia were studied. These are: (1) a new genus and species in *Aedes aegypti* having developmental sequences intermediate between *Amblyospora* and *Tuzetia*, (2) *Polydisprenia caecorum* in *Culex quinquefasciatus* and *Culiseta inornata* and (3) *Hazardia milleri* in *Cx. quinquefasciatus*. All but one (*H. milleri*) were found to undergo meiosis producing haploid spores. Spores of the first species are infectious to healthy larvae producing gametes that fuse to form diplokarya in young larvae.

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